Cytotoxic Activities of Several Geranyl-Substituted Flavanones

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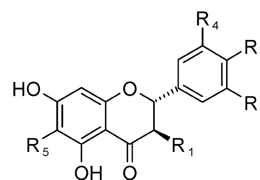
Nine geranylated flavanones isolated from the fruits of *Paulownia tomentosa* (**4**–**12**) and two from the roots of *Morus alba* (**13** and **14**) were examined for cytotoxicity to selected human cancer cell lines and normal human fibroblasts. Cytotoxicity was determined in vitro using a calcein AM cytotoxicity assay. Cytotoxicity for the THP-1 monocytic leukemia cell line was tested using erythrosin B cell staining. The geranylated compounds tested were compared with the known simple flavanone standards taxifolin (**1**), naringenin (**2**), and hesperetin (**3**) and with the standard anticancer drugs olomoucine II, diaziquone, and oxaliplatin and the antineoplastic compound camptothecin, and showed different levels of cytotoxicity. The effects of structural changes on cytotoxic activity, including geranyl substitution of the flavanone skeleton and the oxidation pattern of ring B of the flavanones, are discussed.

Polyphenolic compounds occur in nature as secondary metabolites in many plant species. According to their basic skeleton, they can be divided into several categories with one common structural feature, namely, the presence of phenolic hydroxy groups. Aromatic organic acids, coumarins, phthalides, quinones, stilbenes, xanthones, and especially flavonoids are included.[1] The biological activities of plant phenols vary and are often modified by the presence of different substituents on the basic skeleton. One possible structural modification is substitution by a prenyl side chain.

Prenylated flavonoids resulting from the combination of the phenolic and terpenoid metabolic pathways are often the focus of phytochemists and experimental biologists because of their interesting chemical properties and biological activities. The structural characteristic of prenyl flavonoids combines the lipophilic properties of prenyl or geranyl side chains with the hydrophilicity of the phenolic skeleton. The type of prenyl substitution and other modifications affect the biological activity of modified phenolic compounds.[2–4] Prenylated phenols can exhibit a broad spectrum of biological effects, including antioxidative, antiphlogistic, anticarcinogenic, and estrogenic properties, as described, for example, for the prenyl xanthones[5] and for prenyl compounds isolated from hops.[6,7] The ability of prenylated phenols to inhibit growth significantly or to induce cell death of bacteria and cancer cells arises from this wide spectrum of biological activities. The antibacterial activity of prenylated chalcones,[8] prenyl flavonoids,[9] or pterocarpans,[10] the effect of prenylation on the cytotoxic potential of flavonoids,[2,3,11,12] the cancer-related activities of prenylated phenols derived from hops,[13] and the potent cytotoxic properties of prenylated stilbenes[14] have been described previously. Due to the increasing interest in geranylated flavanones and their possible biological effects in cancer treatment and their antibacterial effects, we isolated eight geranylated flavanones (**4**–**9**, **11**, and **12**) from fruits of the Chinese medicinal plant *Paulownia tomentosa* Baill. (Scrophulariaceae).[15] We isolated subsequently and identified another geranylated substance called schizolaenone C (**10**), previously detected only in the fruits of *Schizolaena hystrix* Capuron (Sarcolaenaceae), from *P. tomentosa*.[16] Two *C*-geranyl flavanones (**13** and **14**) were obtained by HPLC fractionation of a root extract of *Morus alba* L. (Moraceae), as described in this work. On the basis of the analysis of spectroscopic data and comparison with published values, compound **13** was identified as kuwanon E.[17] Compound **14**, 4′-methoxykuwanon E, was isolated as a new compound.

On the basis of previous findings, the cytotoxicity of flavanones **4**–**14** was tested against five human cancer cell lines and on normal human fibroblasts using the calcein AM cytotoxicity assay. Cell viability and cytotoxicity were established also by staining THP-1 monocytic leukemia cells using erythrosin B. The cytotoxic effects were then compared with those of the known simple nongeranylated flavanones taxifolin (**1**), naringenin (**2**), and hesperetin (**3**), as well as the standard anticancer drugs olomoucine II, diaziquone, and oxaliplatin, and the antineoplastic lead compound camptothecin.



	R_1	R_2	R_3	R_4	R_5
1	OH	OH	OH	H	H
2	H	H	OH	H	H
3	H	OH	OMe	H	H
4	OH	OMe	OH	H	geranyl
5	H	OMe	OH	H	geranyl
6	H	OMe	OH	OMe	geranyl
7	H	OMe	OH	OH	geranyl
8	H	OH	OH	H	geranyl
9	H	H	OH	H	geranyl
10	H	OH	H	OH	geranyl

Results and Discussion

Compounds **4**–**9**, **11**, and **12** were isolated from *P. tomentosa* as reported recently,[15] with compound **10** obtained as described in the Experimental Section. Compounds **13** and **14** were isolated as

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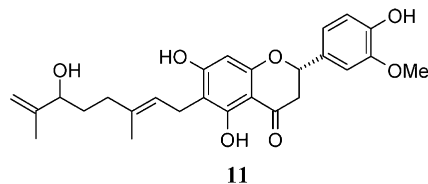
brownish, amorphous powders from the $CHCl_3$ fraction of *M. alba* extract by exhaustive HPLC fractionation. Compound **13** was determined to be identical with a geranyl flavanone previously isolated from *M. alba* and assigned as kuwanon E.[17] Circular dichroism (CD) spectroscopic analysis and comparison with previous results were used to establish the absolute configuration at the C-2 stereogenic center of **13**.[15] The absence of a Cotton effect for $n \rightarrow \pi^*$ electronic transitions at ca. 340 nm and for $\pi \rightarrow \pi^*$ at ca. 290 nm led to the conclusion that **13** was obtained as a racemic mixture.

is a crucial structural requirement for the cytotoxic effect of flavanones on the cancer cell lines tested. Comparison of the EC_{50} values of compounds **5**, **11**, and **12** demonstrated that hydroxylation of the geranyl substituent in compounds **11** and **12** decreased the cytotoxic activity for all lines tested. A previous study has also shown reduced cytotoxicity for similar flavanone compounds with the *C*-geranyl side chain modified by hydroxylation.[25] The effect of the position of the *C*-geranyl group was clearly visible when the cytotoxicity of compounds **8**, **10**, **13**, and **14** was compared. A significant increase in activity was observed when the *C*-geranyl side chain occurred at position 6 on the A-ring (**8** and **10**), when compared to position 3′ in the B-ring (**13** and **14**) of the flavanone skeleton.



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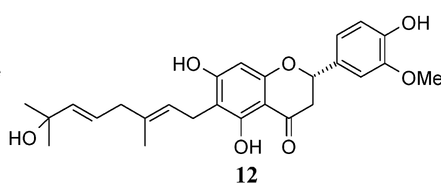

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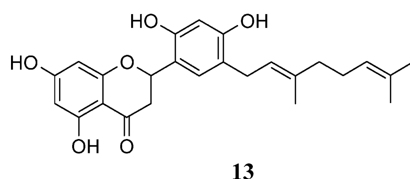

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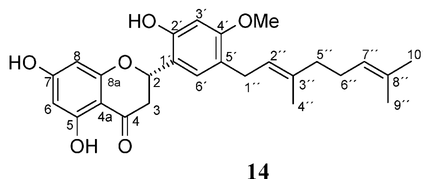

14

Compound **14** was isolated as a new compound, and its structure elucidation is described. The structure of compound **14** was determined to be 4′-*O*-methylkuwanon E. On the basis of HRAP-ITOFMS analysis ($[M - H]^-$ *m/z* 437.1867), the molecular formula of **14** was established as $C_{26}H_{30}O_6$. Chemical shifts similar to those obtained for **13** were observed in the ^1H and ^{13}C NMR spectra of **14** (Table 1). However, several minor differences observed between **13** and **14** are highlighted below. In the ^1H NMR spectrum, a signal was detected for a methoxy group at C-4′ (δ 3.73 ppm, 3H). The HMBC experiment confirmed the presence of this structural feature from interactions of C-4′ with H-1″ and CH_3O. The assignments of protons H-3α and H-3β were analogous to those obtained for compound **13** [H-3α (*cis*): δ 2.62 ppm, J = 3.1 Hz; H-3β (*trans*): δ 3.24 ppm, J = 12.8 Hz)]. A positive Cotton effect for the $n \rightarrow \pi^*$ electronic transition at ca. 330 nm and a negative Cotton effect for the $\pi \rightarrow \pi^*$ electronic transition at ca. 290 nm were observed in the CD spectrum of **14**. Therefore, a 2*S* configuration was assigned to this compound.[15]

Previous reports have described some effects of flavanones in the prevention of cancer development and their cytotoxicity for cancer cell lines.[18] For *C*-geranyl compounds, for example, analysis of propolis from Taiwan and Brazil showed the presence of cytotoxic geranyl flavanones.[19] Propolin C [a synonym for diplacon (**8**)] has been demonstrated to be cytotoxic to human melanoma cells and to be a potent trigger of apoptosis.[19] Propolin H showed similar properties in a subsequent study by Weng et al.,[20] while propolin G induced apoptosis in brain cancer cell lines.[21] A *C*-geranyl compound with an unsubstituted flavanone B-ring showed some activity against the human pancreatic cancer cell line PANC-1.[22] Several geranyl flavanones isolated from hops induced apoptosis in the human Burkitt's lymphoma BJAB cell line.[23]

Therefore, the flavonoid compounds isolated from *P. tomentosa* (**4**−**12**) and *M. alba* (**13** and **14**) were tested against seven different human cell lines: breast carcinoma (MCF-7), T-lymphoblastic leukemia (CEM), multiple myeloma (RPMI 8226 and U266), cervical cancer cells (HeLa), THP-1, a monocytic leukemia cell line, and a normal BJ fibroblast cell line. Six different concentrations of the compounds were used to obtain EC_{50} values, which were comparable with previous results (Table 2).[24,25] Evaluation of the EC_{50} data showed that the geranylated derivatives exhibited significantly greater cytotoxicity than the corresponding simple flavanones. This suggests that the presence of a geranyl side chain

The effect of the presence of a hydroxy group at position C-3 of ring C was compared for compounds **4** and **5**. The present results confirmed previous reports indicating that OH-3 substitution of flavanones reduces their cytotoxicity for almost all cell lines.[25] Consequently, the effect of substitution of flavanone ring B was compared for compounds **4**−**10**, **13**, and **14** on cancer cell cytotoxicity in order to investigate the differing sensitivities of the cell lines to the compounds. For the MCF-7 line, compounds **7** (OCH_3-3′, OH-4′, OH-5′) and **10** (OH-3′, OH-5′) displayed the least potent cytotoxicity. The CEM line showed the highest sensitivity to **8** (3′,4′-di-OH, EC_{50} 3.2 μM). This was followed by **9** (OH-4′,

Table 1. NMR Spectroscopic Data for Compound **14** at 303 K

position	δ_C	δ_H (*J* in Hz)
2	73.9	5.61 dd (3.0, 12.7)
3	41.1	2.62 dd (3.1, 17.1)
		3.24 dd (12.8, 17.1)
4	196.6	
4a	101.7	
5	163.6	12.14 (OH)
6	95.8	5.86 dd (2.1, 4.5)
7	166.7	9.0 brs
8	95.0	5.86 dd (2.1, 4.5)
8a	163.3	
1′	115.9	
2′	153.9	9.0 brs
3′	99.0	6.47 s
4′	157.7	
OCH_3-4′	55.3	3.73 s
5′	119.7	
6′	127.6	7.11 s
1″	27.4	3.15 d (7.1)
2″	122.8	5.21 t (6.9)
3″	134.9	
4″	15.9	1.64 s (CH_3)
5″	39.2	1.99 m
6″	26.2	2.03 m
7″	124.1	5.06 t (6.9)
8″	130.7	
9″	17.5	1.53 s (CH_3)
10″	25.5	1.59 s (CH_3)

Table 2. Cytotoxic Activities of Simple and *C*-Geranyl-Substituted Flavanones Isolated from *P. tomentosa* and *M. alba* on Different Cell Lines[a]

	cell line tested, EC_{50}[b] [μM]						
compound	MCF-7	CEM	RPMI8226	U266	HeLa	BJ	THP-1
5	<10	<10	<10	<10	9.2 ± 4.2	<10	<10
7	<10	<10	7.3 ± 2.4	5.5 ± 4.0	7.4 ± 8.0	4.7 ± 2.0	<10
8	<10	3.2 ± 2.3	<10	2.4 ± 5.8	<10	5.9 ± 1.9	<10
10	<10	<10	7.1 ± 0.7	1.9 ± 1.1	6.3 ± 0.5	7.5 ± 1.1	8.5 ± 2.9
olomoucine II	4.6 ± 3.8	8.1 ± 1.7	6 ± 2.5	7.2 ± 3.0	9.9 ± 2.2	5.4 ± 4.5	
diaziquone	4 ± 1.3	0.86 ± 0.7	3.4 ± 2.1	2.8 ± 2.9	6.4 ± 1.8	<10	
oxaliplatin	<10	1.2 ± 1.4	1.7 ± 6.4	<10	<10	<10	
camptothecin							0.16 ± 0.07

[a] The calcein AM assay was used for MCF-7, CEM, RPMI8226, U266, HeLa, and BJ cells, and erythrosin B staining for THP-1 cells. The EC_{50} values and corresponding SD were calculated from the dose−response curves of three independent measurements. Compounds with EC_{50} > 10 μM (or EC_{50} > 50 μM for THP-1) were not cytotoxic in the range of concentrations used in these cytotoxicity assays. [b] Compounds **1−4, 6**, **9**, and **11−14** were inactive (EC_{50} > 10 μM) for all cell lines in which they were evaluated.

EC_{50} 11.6 μM) and **10** (3′,5′-di-OH, EC_{50} 12.3 μM). Compound **8** showed a higher cytotoxicity than olomoucine II. For the RPMI 8226 cell line, the most cytotoxic were compounds **7** (OCH_3-3′, OH-4′, OH-5′, EC_{50} 7.1 μM) and **10** (3′,5′-di-OH, EC_{50} 7.1 μM), which had a cytotoxicity similar to that of olomoucine II. Compounds **8** and **9**, with 3′,4′-di-OH and OH-4′, respectively, exhibited EC_{50} values close to 10 μM. Three compounds, **7** (OCH_3-3′, OH-4′, OH-5′), **8** (3′,4′-di-OH), and **10** (3′,5′-di-OH), showed high cytotoxicity, with EC_{50} values in the range 1.9−5.5 μM for the U266 cell line. It is interesting to note that EC_{50} values of the standards were greater in this case. The HeLa cell line was the most sensitive of those used in the experiments, and compounds **5**, **7**, and **10** showed comparable activities to those of olomoucine II and diaziquone and greater activity than that of oxaliplatin. The BJ human fibroblast cell line was also used for testing; compounds **7**, **8**, and **10** showed the highest cytotoxicities, with EC_{50} values in the range 4.7 to 7.5 μM, again comparable to those of the standards used.

For partial comparison, a different method based on erythrosin B staining was used on the monocyte THP-1 cell line. The antineoplastic compound camptothecin was shown to be more than 50 times as active as **10**, the most cytotoxic geranylated flavanone.

These results partially confirm the results published previously for the A2780 ovarian cancer cell line, for which compounds **8** and **9** showed EC_{50} values of 10 and >10 μM, respectively.[16] In contrast, for the same cancer cell line, Murphy et al.[26] have reported EC_{50} values for **8** and **9** of 5.5 and >10 μM, respectively.

It may be concluded from the present data that selected *C*-geranyl flavanones show cytotoxic activity for cancer cell lines. The geranyl substituent is an important factor for flavanone cytotoxicity and should not be modified by hydroxylation. Compounds having a *C*-geranyl side chain attached to ring B of a flavanone skeleton showed less activity than those with the same chain on ring A. Evaluating the effect of B-ring substitution showed that mono- or dihydroxy substitution of flavanones (**8−10**) led to compounds with the most potent activities; strong cytotoxicity was also displayed by the dihydroxy methoxy-substituted flavanone **7**. Comparison of cytotoxicity against normal cells and against cancer cell lines revealed a very narrow therapeutic window for all of the compounds tested.

Experimental Section

General Experimental Procedures. A JASCO P 2000 digital polarimeter (cell volume 1 mL, 10 cm) was used for optical rotation measurement. UV spectra of samples were recorded in MeOH on a Synergy HT multiplate reader. CD spectra were recorded on a JASCO J-810 spectrometer (MeOH; the molar elipticity θ_λ values are reported). IR spectra were determined using the ATR method on a Nicolet Impact 400D FT-IR spectrophotometer. NMR spectra were recorded using a Bruker Avance 300 spectrometer operating at frequencies of 300.13 MHz (^{1}H) and 75.48 MHz (^{13}C). The spectra were measured in DMSO-d_6 or $CDCl_3$ at 303 K. The ^{1}H and ^{13}C NMR chemical shifts (δ in ppm) were referenced to the signals of the solvent [2.50 (^{1}H) and 39.43 (^{13}C) for DMSO-d_6 and 7.26 (^{1}H) and 77.00 (^{13}C) for $CDCl_3$]. 2D NMR, gs-COSY, gs-HSQC, and gs-HMBC were used to assign the individual ^{1}H and ^{13}C resonances. The HSQC experiment was adjusted for the coupling $^1J_{HC}$ = 150 Hz and the HMBC experiment for long-range couplings of 7.5 Hz. ESIMS results for samples dissolved in MeOH were collected on an Agilent HP 1100 LC/MSD Trap VL Series, using direct infusion with a linear pump (K & D Electronics) at a flow rate of 300 μL min^{-1}. The spectra were collected in the negative mode, with the nebulizing and drying gas N_2 (t = 300 °C) flowing at a rate of 10 L min^{-1}, a nebulizer pressure of 80 psi, and a capillary voltage of 3.5 kV. The full mass scan covered the range from *m/z* 200 to 1500. A Mariner PE Biosystem Workstation (Tekas) with APITOF was used to collect the HRMS. These spectra were collected also in the negative mode. An Agilent 1100 apparatus equipped with a diode-array detector was used for chromatographic semipreparative (Supelcosil ABZ+Plus, 250 mm × 10 mm i.d., particle size 5 μm) and analytical (Supelcosil ABZ+Plus, 150 mm × 4.6 mm i.d., particle size 3 μm) separation. Preparative HPLC was carried out on a LCP 4100 instrument, with loop injection of 100 μL, column block LCO 101, UV detector LCD 2084 (Ecom, CR), and column Supelcosil ABZ+Plus, 250 mm × 21.2 mm i.d., particle size 5 μm.

The simple flavanones **1−3** were acquired from Sigma (Czech Republic). The purity of all compounds always exceeded 95%, as determined by analytical HPLC. The geranylated flavonoids **4−9**, **11**, and **12** used in the cytotoxicity testing were isolated from *P. tomentosa* according to the method described previously.[15]

Plant Material. The roots of *M. alba* (Moraceae) were collected in Konya, Turkey, in April 2007. A voucher specimen (MA-07A) was deposited at the herbarium of the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic. The plant material was identified by Assoc. Prof. Murat Kartal (Faculty of Pharmacy, Ankara University, Turkey) and Assoc. Prof. Petr Babula (Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic). The fruit of *Paulownia tomentosa* was collected in the area of the University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic, during October 2004. A voucher specimen (PT-04O) has been deposited at the herbarium of the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic.

Extraction and Isolation. Schizolaenone C (**10**) was obtained from the MeOH part of fraction K (fraction 197−199)[15] of the *P. tomentosa* extract, using flash chromatography and subsequent semipreparative HPLC. Flash chromatography of fraction K was carried out on silica gel Merck 60 (particle size 0.040−0.063 mm), the mobile phase was composed of C_6H_6−$CHCl_3$−MeOH (v/v/v, 5:85:10, with the addition of 0.1% HCOOH), and 150 mL fractions were collected. Fraction K-8, containing geranylated flavonoids, was subsequently separated by analytical and semipreparative HPLC. Fractions were collected on the basis of the UV detector response at λ = 280 nm. The identity of compound **10** was confirmed by comparison of the ^{1}H and ^{13}C NMR, HRMS, IR, and UV data with those in the literature.[16]

The dried root of *M. alba* (537 g) was crushed into small pieces and then extracted by maceration with ethanol (3 × 5 L). The dried ethanol extract (20 g) was dissolved in 90% MeOH (500 mL), and the

resulting uniform suspension was extracted with 3 × 250 mL of hexane to remove strongly nonpolar compounds. One liter of water was added to the MeOH residue, and the resulting solution was extracted with $CHCl_3$ (3 × 500 mL). The $CHCl_3$ extracts were combined and dried in vacuo to give a brownish mass (15 g). This was dissolved in a sufficient volume of MeOH and separated using reversed-phase preparative HPLC. The gradient elution used 0.2% HCOOH and a mixture of MeCN and MeOH, 8:2 (v/v) (A). The gradient of the mobile phase initially consisted of 20% A and reached 100% A in the 40th minute. Flow rate was 25 mL min^{-1}. Fractions were acquired according to the detector response (λ = 280 nm). After removing the organic solvent and precipitation, fractions with t_R of 22−23 and 26−27 min, respectively, yielded compounds **13** and **14** (138 and 182 mg, respectively).

Schizolaenone C (10): yellowish powder; UV (MeOH) λ_{max} (log ε) 340 (sh) (3.55), 285 (4.08), 235 (sh) (4.10), 210 (4.76) nm; ^{1}H and ^{13}C NMR data in accord with ref 16; HRAPITOFMS [M − H]$^-$ *m/z* 423.1789 (calcd for $C_{25}H_{27}O_6^-$ 423.1807); ESIMS [M − H]$^-$ *m/z* 423.5.

Kuwanon E (13): yellowish powder; UV (MeOH) λ_{max} (log ε) 331 (sh), 288 (4.26), 231 (sh), 201 (4.76) nm; ^{1}H and ^{13}C NMR in accord with ref 17; ESIMS [M − H]$^-$ *m/z* 423.

4′-Methoxykuwanon E (14): brownish powder; $[\alpha]^{25}_D$ −14.8 (*c* 0.29, MeOH); UV (MeOH) λ_{max} (log ε) 201 (4.85), 224 (sh), 288 (4.36), 334 (sh) nm; CD (MeOH) θ_{331} +5904, θ_{291} −24 703, θ_{227} 10 595; IR (ATR) ν_{max} 3318, 2966, 2915, 1636, 1604, 1506, 1449, 1356, 1258, 1154, 1107 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS [M − H]$^-$ *m/z* 437; HRAPITOFMS [M − H]$^-$ *m/z* 437.1940 (calcd for $C_{26}H_{29}O_6^-$, 437.1964).

Calcein AM Cytotoxicity Assay. The cell lines used in the cytotoxicity assays, human breast carcinoma (MCF-7), human T-lymphoblastic leukemia (CEM), human multiple myeloma (RPMI 8226 and U266), human HeLa cervical cancer cells, and human normal fibroblasts (BJ), were purchased from the American Type Culture Collection (ATCC). The cells were grown on Dulbecco's modified Eagle's cell culture medium (DMEM, Gibco BRL) supplemented with 10% (v/v) fetal bovine serum, L-glutamine (0.3 g/L), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cell cultures were maintained at 37 °C in a fully humidified atmosphere containing 5% CO_2. Cells were subcultured two or three times a week using a trypsinization procedure.[27]

To determine the anticancer potential of the individual compounds, their cytotoxic effect on the growth of malignant human cancer cell lines was determined by calcein AM cytotoxicity assays in 96-well microtiter plates. For cytotoxicity estimations, 100 mM stock solutions were prepared in dimethylsulfoxide (DMSO) from each compound and diluted to 15 mM with DMSO. Then, 8 μL of this solution was diluted in 232 μL of distilled water just before use to obtain the first concentration (500 μM) to be applied to the cell cultures. Starting with the 500 μM concentration, six 3-fold dilutions of the intended test concentration (ranging from 2 to 500 μM) were prepared by adding 160 μL of distilled water to 80 μL of the solution with the corresponding higher concentration. The cell suspensions were counted in a Bürker chamber, diluted in DMEM to an approximate density of 1.25 × 10^5 cells/mL, then placed into each well of a 96-well plate and stabilized for 3 h. Afterward, six serial 3-fold dilutions of the tested compounds were added to the appropriate microtiter plate wells already containing 80 μL of the cell suspension in 20 μL aliquots. Each concentration tested was added in triplicate; the highest final concentration in the wells was 100 μM and the lowest 0.4 μM. The final concentration of DMSO in the reaction mixture never exceeded 0.6%. After 72 h of incubation at 37 °C and 5% CO_2, the addition of 100 μL of 2 μM calcein AM solution (Molecular Probes) to the microtiter plate wells and incubation for 1 h resulted in the staining of viable cells. The fluorescence of these viable cells was measured and quantified at 485/538 nm (ex/em) using a Fluoroscan Ascent (Labsystems) reader. Each compound was characterized by an EC_{50} value, the concentration of the compound lethal to 50% of the tumor cells, as calculated from the dose−response curves obtained. Calculations were performed using MS Excel 2000.[27]

Cellular Proliferation and Viability Assay. Human monocytic leukemia THP-1 cells were obtained from the European Collection of Cell Cultures (ECACC). These cells were routinely cultured in RPMI medium supplemented with 10% fetal bovine serum, 2% L-glutamine, and 1% penicillin and streptomycin, at 37 °C with 5% CO_2. The compounds tested were dissolved in DMSO and added in five increasing concentrations to the cell suspension in culture medium. Subsequently, the cells were incubated for 72 h at 37 °C with 5% CO_2. The cell number and viability were determined following staining with erythrosin B (Sigma, Germany). The staining solution (0.1% erythrosin B (w/v) in phosphate-buffered saline (PBS), pH 7.2−7.4) was mixed with an equal amount of cell suspension, and the number of viable and nonviable cells was counted manually using a hemocytometer and a light microscope. Cells that remained unstained were considered viable and light red cells as nonviable. The cytotoxic EC_{50} concentrations of the compounds tested were determined by combining the data from the equation generated by the KURV+ Version 4.4b software with statistical analysis using STAT+ software (used for the EC_{50} data derived from the values plotted on the graph). The two methods were compared, and the final EC_{50} values obtained.

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Supporting Information Available: NMR spectra for compound **14** and table of HMBC and NOESY for this substance. This information is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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